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Seasonal Dynamics of Bacterial Colonization of Cotton Fiber and Effects of Moisture on Growth of Bacteria within the Cotton Boll

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A highly replicated 3-year field study was conducted to determine the seasonal patterns of bacterial colonization of cotton fiber from the time of dehiscence of the bolls (the point at which the bolls just begin to open) through harvest and commercial ginning. Bacterial numbers on fiber samples from 16 plots were determined by dilution pour plating with tryptic soy agar containing cycloheximide, and numbers of gram-negative bacteria were determined by plating on tryptic soy agar containing vancomycin and cycloheximide. Populations of bacteria varied from year to year, but in all three seasons the pattern of colonization was generally a pattern consisting of a rapid increase following opening of the bolls and a more or less stable number thereafter throughout the growing season. Gram-negative bacteria accounted for 50% or more of the recoverable bacterial population. We hypothesized that the luxuriant bacterial flora developed as a result of the availability of sufficient free water in the bolls to allow bacterial proliferation with the carbon sources remaining after fiber maturation. Therefore, laboratory experiments were conducted to determine the threshold moisture level allowing growth of bacteria on fiber in the bolls. Bacterial proliferation occurred when as little as 2% moisture was added to air-dried fiber. Using simulated bolls, we demonstrated bacterial growth resulting from dew formation on fiber held in controlled-humidity chambers.

Byssinosis is a respiratory ailment which affects mill workers who respire cotton dust during the course of fiber and textile processing (12). The condition is thought to have several causative factors, one of which is the inhalation of bacteria, or their products, associated with the dust. One component linked to the development of symptoms of byssinosis is the endotoxin of the cell walls of gram-negative bacteria (2, 12). Since field-grown cotton is generally contaminated with bacteria and other microorganisms (for example, see references 15 and 16), it follows that attempts to minimize the exposure of workers to the agents eliciting byssinotic symptoms might include reducing the microbial contamination of cotton fiber. If colonization of the fiber occurred as a result of some manageable factor in the field, it might be possible to intervene in the colonization process or to harvest prior to the outbreak of heavy contamination if it were to occur late in the season after boll maturation and fiber emergence. The present study was undertaken as part of a multilocation investigation to determine the seasonal dynamics of bacterial colonization of cotton fiber in the field. Field experiments were also conducted by other investigators in Starkeville, Miss.; Lubbock, Tex.; and Fresno, Calif. In a highly replicated study we examined the abundance of recoverable aerobic heterotrophs and the fraction accounted for by gram-negative bacteria on fiber throughout three consecutive growing seasons in College Station, Tex., and conducted experiments to determine the effects of in-boll moisture content on the proliferation of fiber-associated bacteria. To our knowledge, this is the first study to document the seasonal pattern of bacterial colonization of cotton fiber in the field for three consecutive seasons with a high intensity of sampling. This study differs from many previously published reports in that populations were enumerated immediately after collection from the field rather than by using commercially harvested, ginned fiber from bales.

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MATERIALS AND METHODS

Soil and agronomic practices. All field experiments were conducted at the research plantation of the Texas Agricultural Experiment Station located in College Station. The soil is a Weswood silt loam (fluventic Ustochrept; fine-silty, mixed, thermic). While the soil pH varies from site to site, it ranges from 7.8 to 8.0, and the soil is calcareous. The cation exchange capacity is on the order of 11 cmol(+) kg^{-1}, and the organic matter content is approximately 0.8 to 1.5%.

Cotton was grown by using conventional agronomic practices for the crop and the region. In 1986, cultivar Stoneville 112 was planted on 15 April, and the lint was harvested on 26 September. In 1987 and 1988, cultivar Stoneville 825 was planted on 21 and 8 April, respectively, and the lint was harvested on 22 and 2 September, respectively. Sixteen plots were included in each year of the study, and they were laid out in an 8-by-2 arrangement. In 1986, the plots were four rows (approximately 3.5 m) wide and 90 m long. In 1987 the plots were 8 rows (approximately 6 m) wide and 8 m long, and in 1988 they were four rows (approximately 3 m) wide and 8 m long. In all 3 years of the study, unopened bolls (just before the period when the bolls began to crack) on the lowermost fruiting branches were tagged with fluorescent surveyor's tape in order to mark them for later collection throughout the season. On each collection date (usually once per week), five individual bolls from each plot (80 bolls total for each collection) were picked and placed individually in paper bags. The bagged bolls were placed in a large plastic zip-seal bag and transported to the laboratory or to the ginning facility. In 1986 all bolls were ginned by hand in the

laboratory by using aseptic procedures. In 1987 and 1988, a small, eight-saw, mechanical gin was used to separate the seed from the lint. The gin was thoroughly cleaned between samples by using high-pressure compressed air. In 1987 only, bolls were collected from the lower fruiting branches (25 to 40 cm above the soil; the horizontal samples) as well as the upper branches of the plant (40 cm and above; the vertical samples) in order to compare the bacterial populations in bolls from these locations.

Fiber moisture content. The moisture content of the ginned fiber was determined by weighing samples of the moist fiber, drying the fiber at 60°C for 48 h, transferring the samples to a desiccator, and weighing the samples immediately after removal from the desiccator. The moisture contents are reported below on a percentage (weight/weight) basis.

Bacteriological analyses and endotoxin content of fiber. To determine the numbers of recoverable aerobic, heterotrophic bacteria and the numbers of gram-negative, aerobic, heterotrophic bacteria on fiber, samples of lint were placed in phosphate buffer (pH 7.2) (FTA buffer; BBL Microbiology Systems) containing 0.1% (wt/vol) Tween 80 and shaken at 200 rpm for 20 min on a rotary shaker. Samples (1 ml) from the original dilution were subjected to decimal dilution, and the samples were plated (three plates per dilution) on tryptic soy agar containing cycloheximide (400 $\mu g\ ml^{-1}$) to determine the numbers of total bacteria and on tryptic soy agar containing cycloheximide plus vancomycin (15 $\mu g\ ml^{-1}$) to determine the numbers of gram-negative bacteria. Aliquots (0.1 ml) were spread on the agar surfaces, and the plates were incubated for 48 to 72 h prior to counting of the colonies. A second inspection of the plates was made after 7 days. A study on the ability of the medium containing vancomycin to eliminate gram-positive bacteria showed that approximately 90% of the colonies forming on this medium were gram-negative bacteria, as revealed by Gram staining of more than 100 colonies from dilution plates. The remaining bacteria were gram positive or gram variable, and most appeared to be coryneforms. Analysis of the fatty acid methyl ester profiles of some of these isolates revealed that they were members of the genus *Clavibacter*.

The endotoxin analyses were conducted in the laboratory of Janet Fischer at the University of North Carolina, Chapel Hill. Endotoxin was determined by using the *Limulus* amoebocyte lysate assay.

Bacterial populations on bracts. Bacteria on bracts were enumerated by using the methods described above for fiber populations. However, all bracts from the 80 bolls ginned for analysis of fiber populations were pooled, and three 1-g replicates were subsampled for analysis of bacteria.

Effects of moisture on growth of fiber-associated bacteria. To determine the moisture content at which bacterial growth could occur on fiber, we conducted two types of experiments. In the first, we added various quantities of water to air-dried fiber by misting the fiber with an atomizer. One-gram fiber samples were placed on sterile foil on the pan of a top-loading balance, and the fiber was sprayed to achieve a range of moisture contents on a weight basis. Following the addition of water the fiber was incubated as a simulated boll (see below) in a sealed Mason jar. In order to determine the effects of dew formation on bacterial proliferation, we incubated nonsterile fiber in simulated bolls. In these studies, simulated bolls (1 g of air-dried fiber) were incubated in jars containing known salt solutions to poise the relative humidity (RH) at 75% (saturated NaCl) or 95% (saturated KH_2PO_4) (17, 18). Samples were held at a constant temperature of 30°C for 3 days or were subjected to several (two or three) shifts between 30 and 15 or 18°C at 12-h intervals to simulate day-night temperature shifts and to promote the formation of dew on or within the simulated bolls enclosed in the jars. We prepared simulated cotton bolls by placing 1 g of air-dried (37°C) fiber into a cylindrical cage (diameter, 5 cm; height, 5 cm) made from fiberglass window screen. All components of the apparatus were autoclaved prior to use. The screen cage was suspended on a short piece of nichrome wire attached to the lid of a Mason jar. In this manner, the fiber never made contact with condensation forming on the walls of the jar or with the solution in the bottom. Treatments were replicated six times. Bacteria were enumerated by plating them on 0.25× tryptic soy agar. Baseline populations were determined by enumerating the bacteria in three 1-g samples at the initiation of each experiment.

Statistical analyses. The data were analyzed by using Statistical Analysis System procedures for analysis of variance and *t* tests. Means were separated by Duncan's new multiple-range test or the least-significant-difference test. Where differences are reported below, they are at the 95% confidence level ($P = 0.05$). Bacterial numbers were subjected to a $\log_{10}$ transformation prior to statistical analysis.

RESULTS

Rainfall data and fiber moisture contents are summarized in Fig. 1. The cumulative rainfall amounts were 32, 11, and 6.5 cm during the 1986, 1987, and 1988 sampling periods, respectively. In 1986 8 cm of rain fell during the first 20 days of the sampling period, whereas in 1987 and 1988 rainfall amounts during the first 20 days were 3 and 5.2 cm, respectively. In 1986 rainfall events occurred frequently throughout the sampling period, whereas in 1987 rainfall occurred mainly in the second one-half of the sampling period; in 1988 rainfall occurred early, and then very little rain fell for the remainder of the season. It is noteworthy that there was no rainfall until 16 days after boll opening in 1987.

Fiber moisture contents were high immediately after boll opening and declined to values generally between 6 and 10% when the bolls were not collected after a significant rainfall event. Rain on the day or evening prior to collection had the obvious effect of increasing the moisture content of the fiber. It should be pointed out however that even on mornings when there was no significant rainfall preceding collection, the bolls were wet to the touch, which was also evidenced by wetting of the paper bags in which the bolls were placed. The moisture presumably resulted from condensation within the bolls or from water dripping off dew-soaked leaves. The moisture contents reported are the moisture contents of the ginned fiber, and there was undoubtedly some drying of the fiber as it went through the ginning process, especially when fiber was ginned mechanically. The greater moisture contents of the samples from 1986 may have been a result of hand ginning rather than mechanical ginning, which subjected the fiber to a much greater degree of drying because of the airflow in the gin.

It should also be pointed out that the cotton plants in 1986 were much taller (approximately 2 m) than the cotton plants in 1987 and 1988 (approximately 1 m). Thus, in 1986 the lower portion of the canopy was subject to greater moisture levels and experienced less airflow and hence less evaporative drying than plants in a more open, shorter-statured canopy (as occurred in 1987 and 1988). The foliage in the canopy was always very wet at the time that the bolls were collected (7:30 to 8:00 a.m.).

Bacterial populations. The data from the 3-year study of

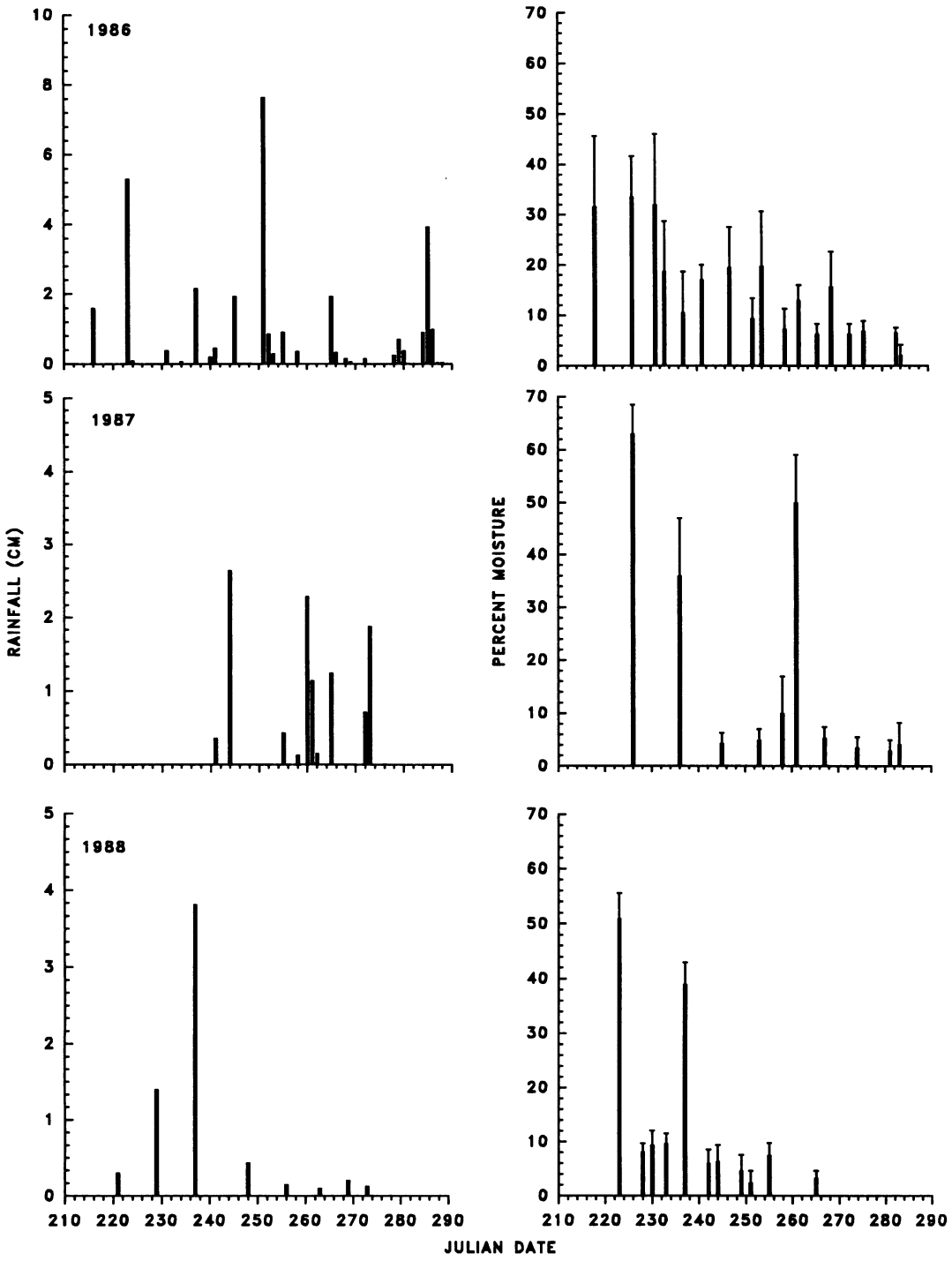

FIG. 1. Rainfall at the Texas A&M University farm for the years 1986 through 1988 and moisture contents of ginned fiber used in plate counts of bacteria. The weather station was located within 100 m of the experimental plots.

bacterial populations associated with fiber are summarized in Fig. 2. Endotoxin data are also plotted in this figure. There was a very distinctive rapid increase in bacterial numbers following the opening of the bolls. Bacterial numbers rose to values between 10^6 and 10^8 CFU g^{-1} and remained at these levels throughout the season. Increases in numbers in response to wetting by rainfall were apparent on some occasions (for example, the spike occurring after the rain on Julian date 260 in 1987 [Fig. 2]). Population numbers were characteristically relatively stable throughout the season, although there was clearly variation in the numbers on different dates. Gram-negative bacteria generally accounted for the majority of the bacteria enumerated (range, 60 to 90% of the bacteria counted). There were also noticeable shifts in

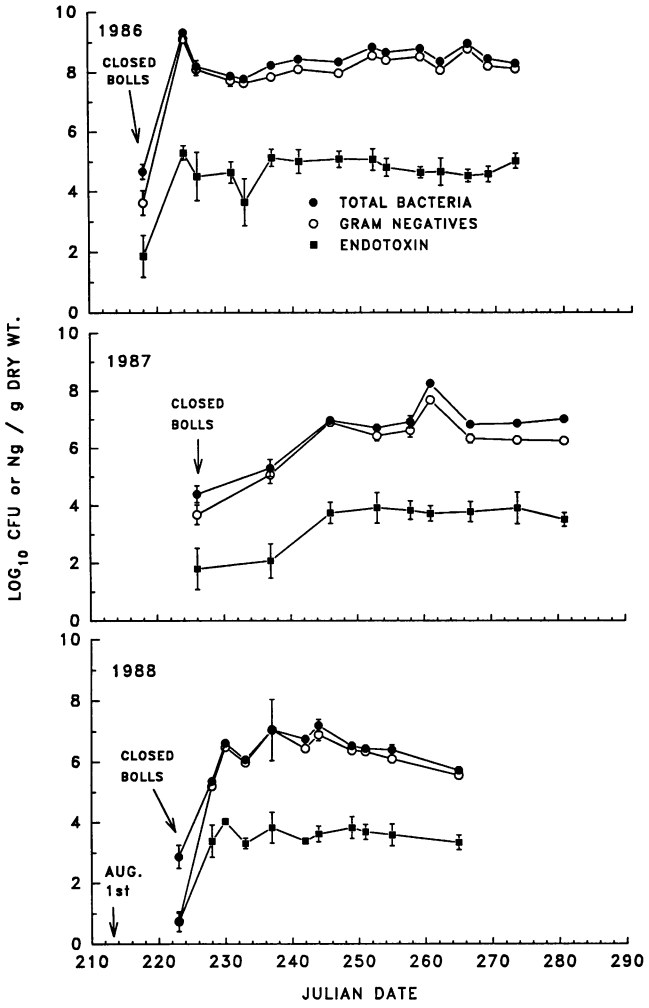

FIG. 2. Bacterial populations and endotoxin contents of cotton fiber samples collected at College Station, Tex. The first point in each curve represents the value for closed bolls; each point thereafter represents fiber from open bolls. Data are the means of results from 16 plots.

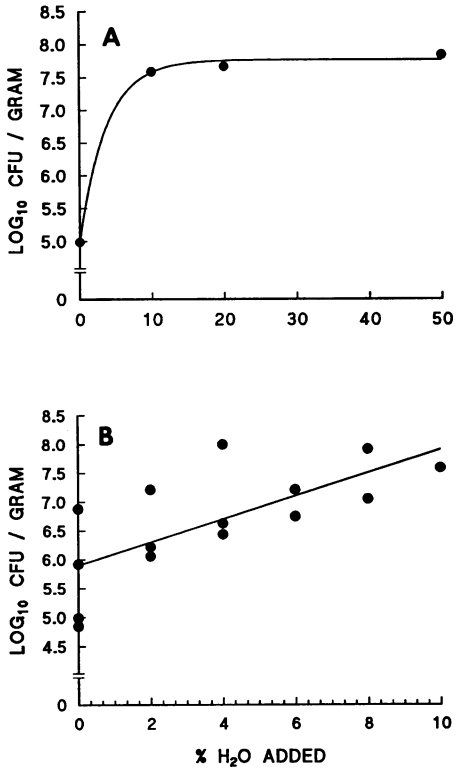

FIG. 3. Effects of added water on growth of bacteria associated with ginned cotton fiber. The data points are means from three replications. Moisture was added by spraying the fiber with sterile water.

species composition, as evidenced by colony morphologies, and there was a trend for fungal populations to increase in the later part of the growing season.

In 1987, bolls from the lower portions of the plants (fruiting branches 1 and 2) were compared with bolls from the remainder of the canopy. The populations on the upper bolls were significantly lower than those on the lower fruiting branches, as revealed in a t test ($P = 0.05$) based on the means of the pooled data; however, the seasonal means of the two populations differed by only 0.14 log (data not shown). The horizontal and vertical samples tended to vary together, and it seems doubtful that any statistical separations of the two samples are actually biologically relevant.

The endotoxin values tended to follow the same trend as bacterial numbers, although there were some points at which increased bacterial numbers were not reflected in increased endotoxin contents. In 1986, the bacterial populations were greater than in the two subsequent years, and this was reflected in the endotoxin contents as well.

The bacterial numbers on bracts were generally 1 to 2 orders of magnitude greater than the bacterial numbers recovered from fiber and tended to increase through the season as the bracts became weathered and decomposed (data not shown).

Effects of moisture and humidity on growth of bacteria. The effects of added moisture on growth of fiber-associated bacteria are shown in Fig. 3. The results from one of the preliminary experiments in which up to 50% H_2O was added to the fiber are shown in Fig. 3A. These experiments indicated that bacterial numbers leveled off when more than approximately 10% H_2O was added. Moisture contents greater than 10% did not significantly increase bacterial numbers. In subsequent experiments the moisture contents of fiber were adjusted to between 0% (fiber dried at 37°C) and 8 to 10%. The results of studies performed at the lower moisture contents suggest that bacterial numbers increased linearly with increasing moisture contents (Fig. 3B), although in one experiment there was a marked increase in bacterial numbers at moisture contents between 0 and 2% and little or no increase thereafter (data not shown). The results of these experiments suggest that the addition of relatively small quantities of water to cotton fiber incubated in simulated bolls led to significant bacterial proliferation.

Results of the humidity experiments are summarized in Fig. 4. Fiber exposed to 75% RH (saturated NaCl) absorbed 0.1 to 6% moisture (Fig. 4a and b). In short-term (48- to 72-h) experiments such as experiment 1 (Fig. 4A) at 75% RH, there was little difference in the populations at 0 and 75% RH, except that the 0% treatment held at a constant temperature of 30°C produced a lower population than the treat-

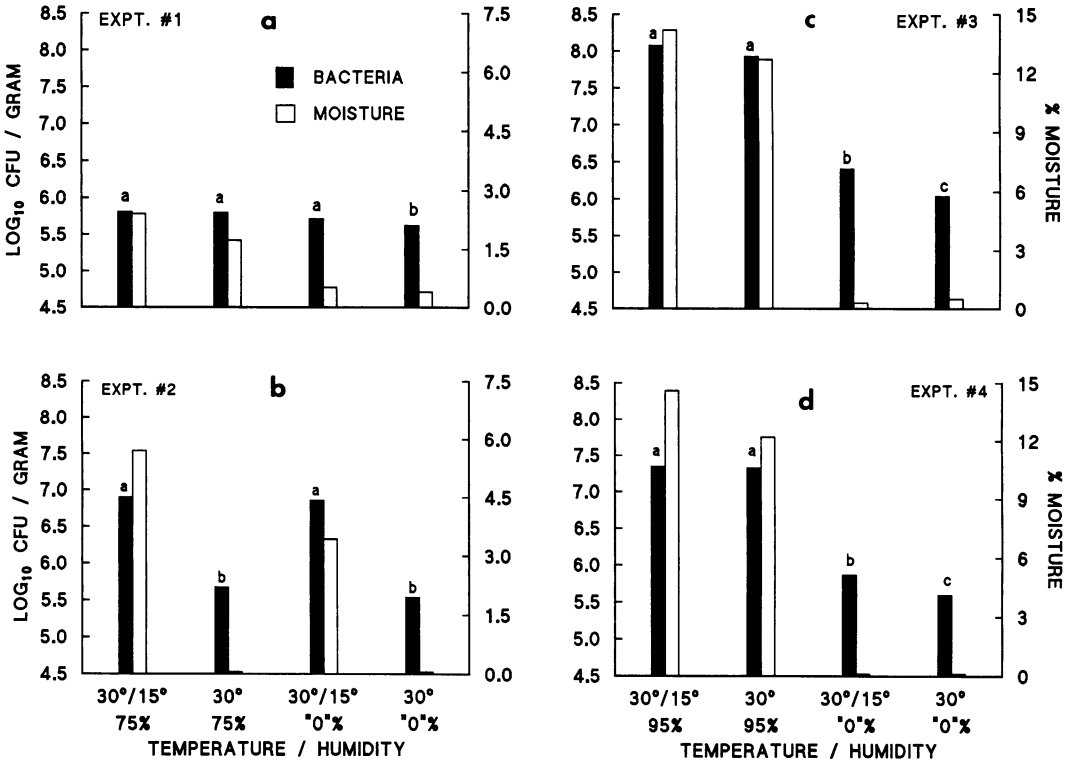

FIG. 4. Numbers of bacteria versus moisture content of cotton fiber held at controlled levels of humidity and cycled between 30 and 15°C to simulate day-night temperature changes. The data are means from three replications. Bars with different letters are significantly different ($P = 0.05$).

ment that was cycled between 15 and 30°C. Fiber moisture content remained below 3% in all treatments. In longer-term experiments (i.e., 5 days) bacterial populations were significantly greater (1 to 1.5 orders of magnitude) in the treatments subjected to temperature cycling than in the treatments maintained at a constant temperature (Fig. 4b). Fiber moisture content attained values between 3 and 6% in the treatments subjected to temperature cycling but remained well below 1% in the treatments held at 30°C (Fig. 4b).

Incubation of simulated bolls at 95% RH (Fig. 4c and d) led to fiber moisture contents ranging from 12 to 15% regardless of temperature treatment, and bacterial numbers were about 2 logs greater than those in the 0% RH treatments, where fiber moisture contents remained below 1%. Whereas there were no significant differences between the temperature treatments at 95% RH, temperature cycling in the 0% RH treatments led to a small but significant increase in bacterial numbers. These results suggest that beyond a certain level of RH sufficient moisture accumulates to allow bacterial proliferation regardless of temperature shifts. However, at lower RH values the temperature cycle appears to contribute to the development of bacteria on the fiber. The indigenous population on the fiber used in all of these experiments was relatively stable and ranged from $\log_{10}$ 4.9 to 5.5 CFU g^{-1}.

DISCUSSION

The results of the 3-year study indicated that cotton grown in College Station, Tex., was rapidly and abundantly colonized by bacteria immediately after the bolls opened. Similar patterns were observed by investigators conducting similar studies at sites in Lubbock, Tex., and Starkeville, Miss. (1, 10) and in separate investigations at New Orleans, La. (5). However, it must be emphasized that the first datum point in each of the graphs of bacterial numbers from this study represents closed or barely cracked bolls. Thus, it was apparent that colonization may have preceded boll cracking for some of the harvested bolls or that colonization of cracked bolls is nearly immediate. Colonization of closed-boll cotton has been reported previously by Millner et al. (14), who found numbers similar to (10^5 to 10^7 CFU g^{-1}) or greater than those found in the present study. Very large populations (up to 10^9 CFU g^{-1}) have also been recovered from closed bolls showing signs of insect damage or bolls frozen on the plants (3).

The pattern of development of the bacterial population was nearly the same in all years of our study. Following boll opening there was usually a rapid increase (within 7 to 10 days) of several orders of magnitude, after which numbers remained relatively constant throughout the remainder of the season. However, in 1987, the numbers rose more gradually, reaching a maximum in 20 days, probably because of a lack of any significant rainfall during this early period. The numbers increased following rainfall events, and on one occasion (Julian date 260) in 1987 fiber was purposefully collected after a heavy rain the previous evening. The numbers associated with this set of samples were greater by 1 order of magnitude. The effects of rain on the numbers of bacteria associated with cotton fiber in the field were similar to the effects reported by Fischer and Sasser (8). The data of Heintz et al. (11) also support the contention that rainfall or even dew formation promotes growth of bacteria on fiber. In

their study, cotton maintained under a rainout shelter developed bacterial populations several orders of magnitude lower than the populations on cotton which was not sheltered. Similarly, cotton from the San Joaquin Valley in California contained very low numbers of bacteria, apparently because of the very low humidity and lack of rainfall to wet the fiber (9a). The greater numbers of bacteria observed in our study in 1986 probably resulted from the more uniform distribution of rainfall and the dense canopy of the 1986 crop compared with the other two seasons, when the plants were shorter.

The lower numbers of bacteria associated with bolls from the upper portions of the plants may have been a result of their being subject to a greater degree of drying because of exposure to sunlight and more air movement. The lower bolls were also exposed to a greater degree of contamination from soil splashed during rainfalls. It was not uncommon to observe visible evidence of soil contamination on fiber in the bolls on the lowermost portions of the plants.

The content of endotoxin, a constituent of the cell walls of gram-negative bacteria, closely paralleled the curves for the abundance of bacteria. Thus, the bacterial count, either total or gram-negative, was a good indicator of the endotoxin load of a given set of samples. The points where endotoxin content and cell numbers do not track together are probably a result of random sample variation.

The bacteria associated with fiber are probably derived from a number of sources, including the bacteria occurring in closed bolls, in wind-borne dust, in dew dripping from leaves and other plant parts, and in soil splashed up from rain that dislodges the soil. All parts of the cotton plant are colonized with bacteria (9), and it is likely that many of the fiber-associated bacteria are derived from other plant parts, especially the bracts, which are rich in bacteria (6, 7, 11; this study). In the present study, the populations associated with the bracts were consistently 1 to 2 orders of magnitude greater than the populations on the fiber (data not shown).

We observed bacterial growth when as little as 2% moisture was added to fiber and then the preparations were incubated for several days. The numbers tended to increase linearly with moisture additions up to 10%, after which there was no further stimulation. It is most noteworthy that relatively small additions of water allowed the breakthrough of bacterial growth. This suggests that the formation of dew on fiber in the field would be sufficient to promote bacterial growth. Thus, it may be that rain per se is not necessary to promote growth of the bacteria, although prevailing evidence (11; this study) indicates that rainfall markedly increases the colonization of fiber. The possibility that humidity and dew formation alone may be sufficient to allow growth of bacteria on fiber was substantiated by the use of simulated bolls in humid chambers. Incubation of simulated bolls at 75 and 95% RH with temperature shifts from 30 to 15°C and back caused the fiber to absorb varying levels of moisture. Moisture accumulation was generally greater when the temperature was changed, although not significantly. However, an accumulation of 2% or greater moisture might be of significance under some circumstances. Humidity influences the survival of bacteria on plant parts (13) and on cotton fiber incubated under controlled conditions (4); thus, it might be expected that moist climates prone to high dew points favor the development and survival of bacteria on fiber throughout the growing season. If this is true, there is probably very little that can be done to minimize the colonization of fiber grown in such locations.

Our results were in general agreement with those of the other investigators conducting similar studies in Mississippi and in Lubbock, Tex. Our study showed conclusively that fiber in the field at College Station was rapidly colonized once the bolls opened and that populations either persisted, turned over at a stable rate, or resulted from continuous recruitment throughout the growing season. This was in sharp contrast to the results obtained from fields in the San Joaquin Valley in California, where very little colonization of the fiber was observed until the cotton was artifically moistened with sprinkle irrigation (9a). Our studies also demonstrated the effects of moisture on bacterial proliferation on fiber incubated under controlled-humidity conditions, and they indicated that relatively low moisture contents may allow bacteria to proliferate on fiber in the field.

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